HER2 (*erb*B-2)-targeted effects of the ϖ-3 polyunsaturated. Fatty acid α-linolenic acid (ALA; 18:3n-3) in breast cancer cells: the «fat features» of the «Mediterranean diet» as an «anti-HER2 cocktail»*

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Background. Data derived from epidemiological and experimental studies suggest that alpha-linolenic acid (ALA; 18:3n-3), the main ϖ-3 polyunsaturated fatty acid (PUFA) present in the Western diet, may have protective effects in breast cancer risk and metastatic progression. A recent pilot clinical trial assessing the effects of ALA-rich dietary flaxseed on tumor biological markers in post-menopausal patients with primary breast cancer demonstrated significant reductions in tumor growth and in HER2 (*erb*B-2) oncogene expression.
Hypothesis. The molecular mechanism by which ALA inhibits breast cancer cell growth and metastasis formation may involve a direct regulation of HER2, a well-characterized oncogene playing a key role in the etiology, progression and response to some chemo- and endocrine therapies in approximately 20% of breast carcinomas.
Methods. Using HER2-specific ELISA, flow cytometry, immunofluorescence microscopy, Western blotting, RT-PCR and HER2 promoter-reporter analyses, we characterized the effects of exogenous supplementation with ALA on the expression of HER2 oncogene, a master key player in the onset and metastasis formation of breast cancer disease. Metabolic status (MTT) assays were performed to evaluate the nature of the cytotoxic interaction between ALA and the humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin®). To study these issues we used BT-474 and SKBr-3 breast cancer cells, which naturally exhibit amplification of the HER2 oncogene.
Results. ALA treatment dramatically suppressed the expression of HER2-coded $p185^{Her\text{-}2/neu}$ oncoprotein as determined by ELISA, flow cytometry, immunofluorescence microscopy and immunoblotting techniques. Interestingly, ALA-induced down-regulation of $p185^{Her\text{-}2/neu}$ correlated with a transcriptional response as no HER2 mRNA signal could be detected by RT-PCR upon treatment with optimal concentrations of ALA (up to 20 μM). Consistent with these findings, ALA exposure was found to dramatically repress the activity of a Luciferase reporter gene driven by the HER2 promoter. Moreover, the nature of the cytotoxic interaction between ALA and trastuzumab (Herceptin®) revealed a significant synergism as assessed by MTT-based cell viability assays.
Conclusions. i) These findings reveal that the ϖ-3 PUFA ALA suppresses overexpression of HER2 oncogene at the transcriptional level, which, in turn, interacts synergistically with anti-HER2 trastuzumab-based immunotherapy. ***ii)*** Our results molecularly support a recent randomized double-blind placebo-controlled clinical trial suggesting that ALA may be a potential dietary alternative or adjunct to currently used drugs in the management of

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HER2-positive breast carcinomas. *iii*) Considering our previous findings demonstrating the «HER2 up-regulatory actions» of the ω-6 PUFA linolenic acid (LA; 18:2n-6) and the «HER2 down-regulatory actions» of the ω-3 PUFA docosahexaenoic acid (DHA; 22:6n-3) and of the ω-9 monounsaturated fatty acid oleic acid (OA; 18:1n-9), it is reasonable to suggest that a low ω-6/ω-3 PUFA ratio and elevated MUFA levels, the two prominent «fat features» of the «Mediterranean diet», should be extremely efficient at blocking HER2 expression in breast cancer cells.

Key words: HER2, *erb*B-2, alpha-linolenic acid, trastuzumab, Herceptin, fatty acids, breast cancer.

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INTRODUCTION

Alpha-linolenic acid (ALA; 18:3n-3) is the main ω-3 polyunsaturated fatty acid (PUFA) present in the Western diet and found in green vegetables such as flaxseed, more commonly known as linseed, and in several vegetable oils including rapeseed and soybean. Data derived from epidemiological and experimental studies suggest that ALA may have protective effects in breast cancer risk and metastatic progression. First, Bougnoux et al[1] reported an inverse correlation between the level of ALA in breast fat and the development of metastasis subsequent to breast cancer treatment. Second, low ALA acid content of adipose breast tissue was associated with an increased risk of breast cancer in a case-control study[2]. Third, diets rich in ALA have been shown to inhibit the growth of spontaneous or carcinogen-induced mammary carcinomas in animals, and the proliferation of human mammary tumor cells *in vitro*, independent of the production of long-chain ω-3 PUFAs[3-7]. Fourth, a randomized double-blind placebo-controlled clinical trial recently demonstrated that ALA-rich dietary flaxseed has the potential to reduce tumor growth in patients with breast cancer by increasing apoptosis and reducing the expression of the HER2 oncogene[8].

At present, HER2 (also called neu and *erb*B-2) represents one of the most important oncogenes in breast cancer. HER2 codes for the $p185^{Her\text{-}2/neu}$ oncoprotein, a transmembrane tyrosine kinase orphan receptor[9,10]. HER2 amplification and overexpression occurs in approximately 20% of invasive breast carcinomas and is correlated with unfavorable clinical outcome[11-13]. Expression of high levels of HER2 is sufficient to induce neoplastic transformation of some cell lines[14,15], suggesting a role for HER2 in the etiology of some breast carcinomas. In this regard, HER2 is overexpressesed and/or hyperactivated in pre-neoplastic breast lesions such as atypical duct proliferations and in ductal carcinoma *in situ* of the breast[16,17]. Moreover, HER2 is a metastatic-promoting gene, enhancing the invasive and metastatic phenotype of breast cancer cells[18,19].

Using HER2-specific ELISA, flow cytometry, immunofluorescence microscopy, Western blotting, RT-PCR and gene reporter analyses, we here assessed the effects of ALA on the expression of HER2 oncogene in breast cancer-derived BT-474 and SK-Br3 cell lines, which exhibit HER2 oncogene amplification and are HER2-dependent[20].

MATERIALS AND METHODS

Cell lines and culture conditions

The human breast cancer cell lines SK-Br3 and BT-474 were obtained from the American Type Culture Collection (ATCC), and they were routinely grown in phenol red-containing improved MEM (IMEM, Biosource International, Camarillo, CA, USA) containing 5% *(v/v)*-heat-inactivated FBS and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

Materials

Alpha-linolenic acid (ALA; 18:3n-3) and vitamin E (*dl*-α-tocopherol) were purchased from Sigma-Chemical (St. Louis, MO, USA). The cultures were supplemented, where indicated, with fatty acid-free bovine serum albumin (FA-free BSA, 0.1 mg/ml) complexed with a specific concentration of ALA. A BSA/ALA concentrated (X100) was formed by mixing 1 ml of BSA (10 mg/ml) with various volumes (1-10 µl) of ALA (200 mg/ml) in ethanol. The concentrate was mixed for 30 min at room temperature before addition to the cultures. Control cultures contained uncomplexed BSA. Trastuzumab (Herceptin®) was kindly provided by the Evanston Northwestern Healthcare Hospital Pharmacy (Evanston, Illinois, USA).

The mouse monoclonal antibodies for HER2-coded $p185^{Her\text{-}2/neu}$ protein (Ab-5 and Ab-3 clones, directed against the extracellular domain and the carboxyl terminal 14 amino acids of $p185^{Her\text{-}2/neu}$, respectively) were from Oncogene Research Products (San Diego, CA). Anti-β-actin goat polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

HER2-specific ELISA

For quantitative determination of $p185^{Her\text{-}2/neu}$, the Human neu Quantitative ELISA System (Oncogene

Science, Bayer Diagnostics) was applied according to the manufacturer's protocol. This immunoassay for detection and quantification of HER2 is sandwich assay utilizing a mouse monoclonal antibody (capture antibody coated onto microtiter wells) and a rabbit polyclonal serum (detector antibody). To assess the effects of ALA on HER2 protein concentration, breast cancer cells, after a 24 h starvation period in media without serum but with 0.5% FA-free BS, were incubated for 48 h with 0.5% FA free-BSA (controls) or increasing concentrations of ALA (10 and 20 μM). 1:5,000-1:10,000 dilutions of total cell lysates from ALA-treated and control untreated SK-Br3 and BT-474 breast cancer cells were used to quantitate HER2 protein expression in cell cultures. The amount of detector antibody bound to antigen was detected using a goat anti-rabbit IgG/horseradish peroxidase conjugate. After the addition of the cromogenic substrate tetramethylbenzidine (TMB), the color range was measured at 492 nm using a microplate reader. A standard curve was generated by using standard solutions as per manufacturer's instructions. The concentration of HER2 in test samples was determined by interpolation of the sample absorbances from the standard curve.

Flow cytometry

Cells were seeded on 100-mm plates and cultured in complete growth medium. Upon reaching 75% confluence, the cells were washed twice with pre-warmed PBS and cultured in serum-free medium overnight. ALA was added to the culture as specified, and incubation was carried out at 37 °C up to 48 hours in low-serum (0.1% FBS) media. After treatment, cells were washed once with cold PBS and harvested by scrapping in cold PBS. The cells were pelleted and resuspended in cold PBS containing 1% FBS. The cells were then incubated with an anti-$p185^{Her\text{-}2/neu}$ mouse monoclonal antibody (clone Ab-5) at 5 μg/ml for 1 h at 4 °C. After this, the cells were washed twice with cold PBS, resuspended in cold PBS containing 1% FBS, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) diluted 1:200 in cold PBS containing 1% FBS for 45 min at 4 °C. Finally, the cells were washed once in cold PBS, and flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton Dickinson, San Diego, CA) equipped with Cell Quest Software (Becton Dickinson). The mean fluorescence signal associated with cells for labeled $p185^{Her\text{-}2/neu}$ was quantified using the GEO MEAN fluorescence parameter provided with the software.

Semi-quantitative RT-PCR

Following treatments with ALA, cells were harvested with Ultraspec™ RNA and total RNA was isolated according to the manufacturer's instructions (Biotecx Laboratories Inc., Madrid, Spain). 1 μg of total RNA was reversed transcribed (RT) and amplified (PCR) by Access RT-PCR System (Promega) using 1 mM of specific primers for HER2 (sense: 5'-GGGCTGGCCC-GATGTATTTGAT-3'; antisense: 5'-ATAGAGGTTGTC GAAGGCTGGGC-3'). As an internal control, β-actin was used. The RT reaction was carried out for 45 min at 48 °C. HER2 and β-actin cDNAs were amplified with 20 cycles to ensure that the PCR reactions were performed in the linear range using the following PCR profile: 96 °C for 30 sec, 60 °C for 1 min and 68 °C for 2 min. The PCR products were separated on a 2% agarose gel and detected by ethidium bromide staining.

Immunoblotting

Following treatments with ALA, cells were washed two times with PBS and then lysed in buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride] for 30 min on ice. The lysates were cleared by centrifugation in an eppendorff tube (15 min at 14.000 rpm, 4 °C). Protein content was determined against a standardized control using the Pierce protein assay kit (Rockford, Illinois, USA). Equal amounts of protein were heated in SDS sample buffer (Laemli) for 10 min at 70 °C, subjected to electrophoresis on 3-8% NuPAGE and then transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose filter paper was minimized by blocking for 1 hr at room temperature (RT) with TBS-T [25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween 20] containing 5% *(w/v)* nonfat dry milk. The treated filters were washed in TBS-T and then incubated overnight at 4 °C with anti-$p185^{Her\text{-}2/neu}$ primary antibody in TBS-T/5% *(w/v)* bovine serum albumin (BSA). The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Labs, West Grove, PA, USA) in TBS-T were added for 1 h, and immunoreactive bands were detected by enhanced chemiluminescence reagent (Pierce, Rockford, Illinois, USA). Blots were reprobed with an antibody for β-actin to control for protein loading and transfer.

In situ immunofluorescent staining

Cells were seeded at a density of 1×10^4 cells/well in a four-well chamber slide (Nalge Nunc International, Rochester, NY, USA). Following treatments with ALA, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100/PBS for 15 min, and stored over-

night at 4 °C with 10% horse serum in PBS. The cells were washed, and then incubated for 2 h with anti-$p185^{Her\text{-}2/neu}$ antibody diluted 1:200 in 0.05% Triton X-100/PBS. After extensive washes, the cells were incubated for 45 min with FITC-conjugated anti-mouse IgG diluted 1:200 in 0.05% Triton X-100/PBS. The cells were washed five times with PBS and mounted with VECTASHIELD + DAPI (Vector Laboratories, Burlingame, CA, USA). As controls, cells were stained with primary or secondary antibody alone. Control experiments did not display significant fluorescence in any case (data not shown). Indirect immunofluorescence was recorded on a Zeiss microscope. Images were noise-filtered, corrected for background, and prepared using Adobe Photoshop.

HER2 promoter activity

Using FuGENE 6 transfection reagent (Roche Biochemicals, Indianapolis, IN) as directed by the manufacturer, overnight serum-starved cells seeded into 24-well plates (~ 5 x 10^4 cells/well) were transfected in low-serum (0.1% FBS) media with 1,500 ng/well of the pGL2-Luciferase (Promega, Madison, WI) construct containing a Luciferase reporter gene driven by an intact HER2 promoter fragment (HER2 wild-type PEA3-binding site-Luciferase) along with 150 ng/well of the internal control plasmid pRL-CMV, which was used to correct for transfection efficiency. After 18 h, the transfected cells were washed and then incubated with either ethanol *(v/v)* or ALA as specified. Approximately 48 h after treatments, Luciferase activity from cell extracts was detected with a Luciferase Assay System (Promega, Madison, WI, USA) using a Victor^{2TM} 1420 Multilabel Counter (Perkin Elmer Life Sciences). The magnitude of activation in HER2 promoter-Luciferase-transfected cells was determined after normalization of the Luciferase activity in cells co-transfected with equivalent amounts of the empty pGL2-Luciferase vector lacking the HER2 promoter (∅-Luciferase) and the internal control plasmid pRL-CMV. This control value was used to calculate the relative change in the transcriptional activities of HER2 promoter-Luciferase-transfected cells in response to treatments after normalization to pRL-CMV. The activity of the wild-type promoter in untreated control cells was defined as 100%.

In vitro chemosensitivity testing

The ability of ALA to modulate breast cancer cell sensitivity to the anti-HER2 monoclonal antibody trastuzumab (Herceptin®) was determined using a standard colorimetric MTT (3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl-tetrazolium bromide) reduction assay. Cells in exponential growth were harvested by trypsinization and seeded at a concentration of ~ 5 x 10^3 cells/200 µl/well into 96-well plates, and allowed an overnight period for attachment. Then the medium was removed and fresh medium along with various concentrations of ALA, 5 µg/ml trastuzumab or combinations of compounds, were added to cultures in parallel. Agents were studied in combination concurrently. Control cells without agents were cultured using the same conditions with comparable media changes. Compounds were not renewed during the entire period of cell exposure. Following treatment, the medium was removed and replaced by fresh drug-free medium (100 µl/well), and MTT (5 mg/ml in PBS) was added to each well at a 1/10 volume. After incubation for 2-3 h at 37 °C, the supernatants were carefully aspirated, 100 µl of DMSO were added to each well, and the plates agitated to dissolve the crystal product. Absorbances were measured at 570 nm using a multi-well plate reader (Model Anthos Labtec 2010 1.7 reader). The cell viability effects from exposure of cells to each compound alone and their combination were analyzed as percentages of the control cell absorbances, which were obtained from control wells treated with appropriate concentrations of the compounds vehicles that were processed simultaneously. For each treatment, cell viability was evaluated as a percentage using the following equation: *(A_{570} of treated sample/A_{570} of untreated sample) x 100.*

Statistical analyses

Data are the mean and 95% confidence intervals (95% CI) of three independent experiments. A two-way ANOVA was used to analyze differences between the treatment and the control groups.

RESULTS

Exogenous supplementation with ALA down-regulates HER2-coded $p185^{Her\text{-}2/neu}$ tyrosine kinase receptor in HER2-overexpressing breast cancer cells

We first performed a quantitative measurement of $p185^{Her\text{-}2/neu}$ levels using a HER2-specific ELISA (fig. 1A). A 48 h exposure of HER2-overexpressing SK-Br3 cells to increasing concentrations of ALA (0, 10 and 20 µM) was found to significantly down-regulate HER2 protein expression by 44% (from 5.24 ± 0.2 fmol HER2 μg^{-1} in untreated control SK-Br3 cells to 2.93 ± 0.3 fmol HER2 μg^{-1} in SK-Br3 cells treated with 20 µM ALA; figure 1A, left panel). An up to 59% reduction in HER2 protein expression was observed in ALA-treated BT-474 cells (from 95% CI = 5.5-6.5 fmol HER2 μg^{-1} in untreated control BT-474 cells to 95% CI = 2.45-2.49 fmol HER2 μg^{-1} in BT-474 cells treated with 20 µM ALA; figure 1A, right panel). Accordingly,

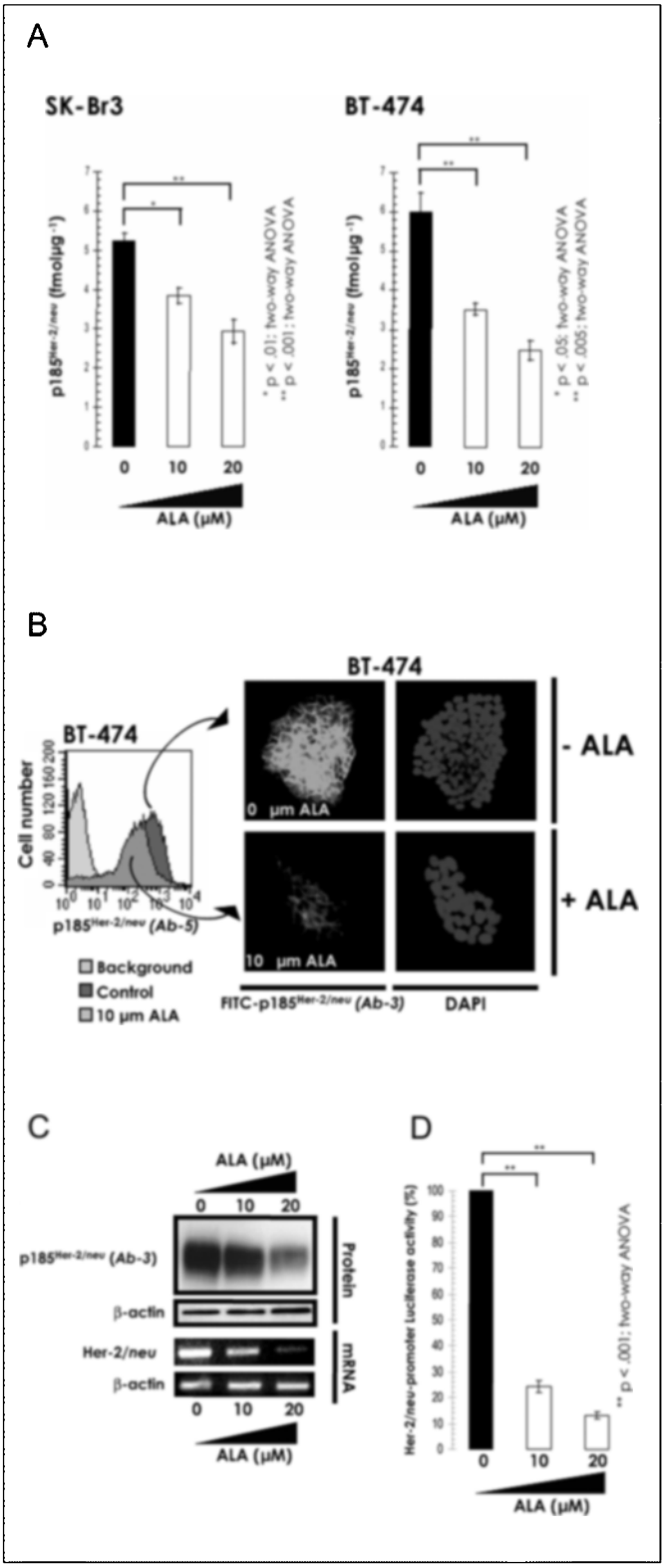

Fig. 1. Exogenous supplementation with ALA down-regulates HER2 expression in breast cancer cells bearing HER2 gene amplification. *A)* The Oncogene Science HER2 Microtiter ELISA was used according to the manufacturer's instructions to compare the baseline expression of HER2 in SK-Br3 *(left)* and BT-474 *(right)* breast cancer cell lines in the presence or absence of increasing concentrations of ALA (0, 10 and 20 μM). *B)* Left. Overnight serum-starved BT-474 cells were cultured in IMEM-0.1% FBS in the presence or absence of 10 μM ALA for 48 h. The specific surface expression of $p185^{Her\text{-}2/neu}$ in ALA-treated cells was determined by flow cytometry by measuring the binding of a mouse anti-$p185^{Her\text{-}2/neu}$ monoclonal antibody directed against the extracellular domain of $p185^{Her\text{-}2/neu}$ (Ab-5 clone) as described in «Material and methods». The mean fluorescence signal ± S. D. (n =3) associated with cells for labeled $p185^{Her\text{-}2/neu}$ was quantified using the Geo Mean fluorescence (GM) parameter provided with the Cell Quest Software (Becton Dickinson) Right. Overnight serum-starved BT-474 cells were cultured in IMEM-0.1% FBS (top panels) or IMEM-0.1% supplemented with 10 μM ALA (bottom panels) for 48 h in eight-well chamber slides. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and labeled for 2 h with an anti-$p185^{Her\text{-}2/neu}$ monoclonal antibody directed against the cytoplasmic domain of $p185^{Her\text{-}2/neu}$ (Ab-3 clone). After labeling, cells were washed thoroughly, and localization of $p185^{Her\text{-}2/neu}$ was detected by indirect immunofluorescence by incubating with FITC-conjugated anti-mouse IgG. After counterstaining with DAPI, cells were examined and photographed using a Zeiss fluorescent microscope equipped with a built-in camera. The figure shows a representative immunostaining analysis. Similar results were obtained in three independent experiments. *C)* Top. BT-474 cells were cultured in the presence of graded concentrations of ALA for 48 h. 20 μg of protein was subjected to Western blot analyses with specific antibodies against $p185^{Her\text{-}2/neu}$ (Ab-3) or β-actin. A representative immunoblotting analysis is shown (n = 3). Bottom. Total RNA from ALA-treated BT-474 cells was isolated and RT-PCR analyses for HER2 and β-actin transcripts expression were performed as described in «Material and methods». The figure shows a representative RT-PCR analysis. Similar results were obtained in three independent experiments. *D)* Overnight serum-starved BT-474 cells seeded into 24-well plates (~ 5 x 10^4 cells/well) were transfected in low-serum (0.1% FBS) media with 1,500 ng/well of the pGL2-Luciferase construct containing a Luciferase reporter gene driven by an intact HER2 promoter fragment along with 150 ng/well of the internal control plasmid pRL-CMV. Experimental treatments and Luciferase activity assays were carried out as described in «Materials and methods». The activity of the ALA-treated samples was calculated relative to that found in control cells treated with ethanol (= 100% Luciferase activity). Data are the mean (columns) and 95% confidence intervals (bars) of three experiments performed in duplicate. One-factor ANOVA was used to analyzed differences in the percentages of reduction in cell viability between the treatment groups. *p < .001 for all the ALA concentrations tested versus control cells treated with *v/v* ethanol (one-factor analysis of variance). All statistical tests were two-sided.

flow cytometric analyses using a monoclonal antibody directed against the extracellular domain of HER2 (Ab-5) established the ability of ALA (10 μM, 48 h exposure) to dramatically decrease the expression levels of cell surface-associated HER2 in BT-474 cells (> 50% reduction; figure 1B, left panel). Equivalent results were found in HER2-overexpressing SK-Br3 breast cancer cells (data not shown).

When we analyzed the impact of ALA treatment in the sub-cellular localization of $p185^{Her\text{-}2/neu}$, untreated cells showed a prominent cell-surface staining of $p185^{Her\text{-}2/neu}$ as assessed by immunfluorescence microscopy. Upon ALA exposure, the membrane staining of $p185^{Her\text{-}2/neu}$ was clearly diminished (fig. 1B, right panel). A significant decrease in the expression of HER2-coded $p185^{Her\text{-}2/neu}$ oncoprotein was also observed in immunoblotting analyses of total protein lysates from ALA-treated BT-474 breast cancer cells using a monoclonal antibody recognizing the carboxyl terminal 14 amino acids of $p185^{Her\text{-}2/neu}$ (Ab-3; figure 1C, top panel).

Exogenous supplementation with ALA reduces HER2 mRNA levels and HER2 gene promoter activity

Although overexpression of HER2 both in tumor and in derived cell lines was originally attributed solely to amplification of *erb*B-2 gene (usually 2- to 10-fold), an elevation in HER2 mRNA levels per gene copy is also observed in all the cell lines examined that exhibit gene amplification[21]. Indeed, an increase in transcription rate sufficient to account for the degree of overexpression has been shown in a number of HER2-overexpressing breast cancer cell lines[22,25]. Our approach therefore sought to characterize the effects of exogenous supplementation with ALA on the transcription rate of HER2 oncogene. First, a dose-dependent reduction on the expression levels of HER2 mRNA was observed in semi-quantitative RT-PCR analyses of RNA isolated from ALA-treated BT-474 cells (fig. 1C, bottom panel). Then, we performed transient transfection experiments with a Luciferase reporter gene driven by the HER2 promoter (pNulit). Remarkably, ALA treatment was found to significantly repress the activity of HER2 gene promoter (up to ~ 80% inhibition) in BT-474 cells. Equivalent results were found in HER2-overexpressing SK-Br3 cells (data not shown). These findings, altogether, strongly suggest that down-regulation of HER2 promoter activity is a major molecular mechanism underlying the anti-HER2 effects observed upon exogenous supplementation with ALA of HER2 gene-amplified human breast cancer cells.

ALA co-exposure synergistically enhances trastuzumab (Herceptin®) efficacy in HER2-overexpressing breast cancer cells

We finally explored whether the down-regulatory effects of ALA on HER2 gene expression could modulate the growth inhibitory effects of trastuzumab (Herceptin®), a humanized monoclonal antibody binding with high affinity to the ectodomain of $p185^{Her\text{-}2/neu}$ oncoprotein and showing encouraging therapeutic effects in patients with HER2-overexpressing metastatic breast cancer[24-28]. We evaluated the change in cell toxicity of a sub-optimal concentration of trastuzumab (5 μg/ml) following a 72 h co-exposure to increasing concentrations of ALA (2.5, 5, 10, 20 and 40 μM ALA). The metabolic status of BT-474 cells cultured with trastuzumab in the absence or presence of ALA was judged by the mitochondrial conversion of the tetrazolium salt, MTT, to its formazan product (MTT-based cell viability assays). The simultaneous presence of ALA during the incubation time with trastuzumab caused an important increase on trastuzumab activity (fig. 2). For instance, while only 13% toxicity was observed in the presence of 10 μM ALA, the cell toxicity induced by 5 μg/ml trastuzumab increased from 14% to 52% (95% CI = 44% to 62%) in the presence of 10 μM ALA. A two-way ANOVA showed that concurrent exposure to ALA and trastuzumab synergistically enhanced the cytotoxic effects achieved

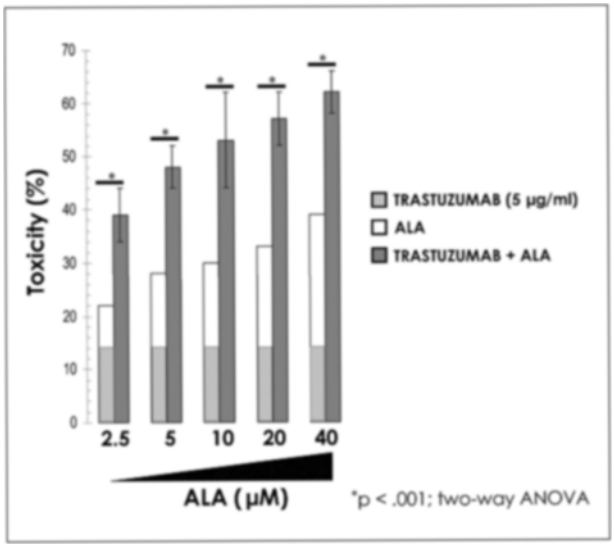

Fig. 2. Concurrent exposure to ALA and trastuzumab synergistically reduces cell viability in HER2-overexpressing BT-474 breast cancer cells. Cell sensitivity to trastuzumab and ALA was determined using a standard colorimetric MTT reduction assay as described in «Materials and methods». For each pair of columns, the height of the columns on the left represents the sum of the effect of each agent alone and, therefore, the expected % reduction in cell viability if their effect is additive when used in combination. The total height of the columns on the right indicates the observed % reduction in cell viability when the agents were used in combination. The difference between the heights of the paired columns reflects the magnitude of the synergy on reducing cell viability. Data are the mean (columns) and 95% confidence intervals (bars) of three experiments performed in triplicate. One-factor ANOVA was used to analyzed differences in the percentages of reduction in cell viability between the treatment groups. *p < .001 for the ALA + trastuzumab groups versus ALA and trastuzumab as single agents for all the ALA concentrations tested (one-factor analysis of variance). All statistical tests were two-sided.

with ALA and trastuzumab as single agents in a statistically significant manner ($p < .001$ for all the range of ALA concentrations combined with trastuzumab).

DISCUSSION

In vitro and animal studies clearly demonstrate that deregulated HER2 expression plays a pivotal role in malignant transformation and tumorigenesis. Overexpression of HER2 has also been associated with more aggressive phenotypes of breast cancer and an increased potential for forming metastases. Although there are controversies regarding HER2 and anti-cancer therapies both in clinical investigations and laboratory studies, substantial evidences have been accumulated indicating that HER2 gene also affects the sensitivity of cancer cells to various treatments such as chemotherapy, hormone therapy, radiation therapy and cytokine treatment. Not surprisingly, patients with HER2-overexpressing breast cancer are associated with unfavorable prognosis, shorter relapse time and low survival rate[29,30].

We here report that exogenous supplementation with ALA specifically suppresses HER2 overexpression at the transcriptional level in cultured HER2 gene-amplified breast cancer cells. ALA-induced repression of HER2 expression, in turn, interacts synergistically with anti-HER2 breast cancer immunotherapy by enhancing the growth inhibitory activity of the anti-Her-2/*neu* monoclonal antibody trastuzumab (Herceptin®) in breast cancer cells with amplification of the HER2 oncogene. Although caution must be applied when extrapolating *in vitro* results into clinical practice, this previously unrecognized property of ALA should help in the understanding of the molecular mechanisms by which individual dietary FAs may regulate the malignant behavior of breast cancer cells. Moreover, a randomized double-blind placebo-controlled clinical trial recently evaluated the effects of dietary flaxseed, which has an exceptionally high concentration of ALA (57% of total fatty acids) on tumor biological markers in postmenopausal patients with newly diagnosed breast cancer[8]. The results of this clinical trial demonstrated that daily intake of 25 g flaxseed can significantly reduce cell proliferation, increase apoptosis, and affect cell signaling by reducing HER2 expression of breast tumors. A 71.0% reduction in HER2 expression and an increase in apoptosis (30.7%) were observed in the flaxseed, but not in the placebo group[8]. Indeed, the total intake of flaxseed was correlated with changes in HER2 expression and apoptotic index[8]. Although the high content of mammalian lignan precursors may also explain some of these effects, our current findings strongly support the notion that ALA is playing a key role in dietary flaxseed-induced reduction in HER2 expression in patients with breast cancer.

It has been assumed that, in contrast to hormones, growth factors and cytokines, gene expression is not affected directly by nutrients. However, it has recently been described that lipids can regulate gene expression[31-33]. Thus, exogenously-derived FAs appear to interact with the human genome by regulating the activity or the amount of transcription factors such as Peroxisome Proliferator-Activated Receptors (PPAR) or the Sterol Response Element Binding Protein (SREBP). We recently considered that these effects of dietary fats on gene expression may open the door to a whole new line of research aimed to molecularly characterize the counterintuitive anti-cancer properties of a high-fat «Mediterranean diet». Recently published experiments from our laboratory showed that dietary FAs previously characterized for either their breast cancer protective effect and/or its tumoricidal actions (ω-3 PUFAs docosahexaenoic acid –DHA- and eicosapentaenoic acid –EPA- and ω-9 monounsaturated fatty acid oleic acid –OA-) significantly down-regulate HER2 expression in human cancer models naturally exhibiting HER2 gene amplification and HER2 protein overexpression[34-39]. Moreover, exogenous supplementation with ALA, DHA, EPA or OA significantly diminishes proteolytic cleavage of the extracellular domain of HER2, a crucial event that determines both the aggressive biological behavior of breast carcinomas and the breast cancer response to chemotherapy, anti-estrogens and the anti-HER2 antibody trastuzumab[34]. Conversely, linoleic acid, a ω-6 PUFA with a strong tumorigenesis stimulating effect, appears to significantly up-regulate HER2 expression (data not shown) while significantly increasing HER2 extracellular domain concentration[34]; (fig. 3). As recently demonstrated by Bougnoux and cols. in their recent hypothesis/commentary «Diet, Cancer and the Lipidome»[40], a careful reappraisal of the role of the environmental exposure to dietary FAs in breast cancer risk (and progression) must be done before public health applications based on some of the components of the so-called «Mediterranean diet». These findings, altogether, underline the concept that a single fatty acid cannot longer be considered as an independent biomarker of breast cancer risk. Alternatively, two prominent fat features of the «Mediterranean diet», i.e., a low ω-6/ω-3 PUFA ratio and elevated MUFA levels appear to be a new composite biomarker of a low risk for breast cancer and, probably, for other types of human cancer. Since HER2 oncogene plays an active role in breast cancer etiology and progression, our current results not only are in line with animal and epidemiological studies showing high levels of ALA to be protective against breast cancer but further provide a novel molecular mechanism underlying the anti-breast cancer (tumoricidal) actions of ALA. Moreover, a low ω-6/ω-3 PUFA ratio and elevated ω-9 MUFA levels, the key "Mediterranean diet's fat

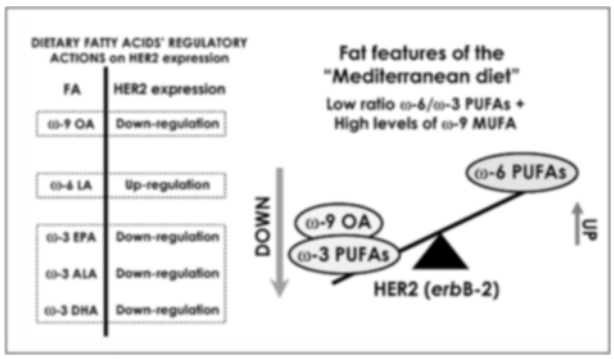

Fig. 3. The «fat features» of the «Mediterranean diet» as an «anti-HER2 cocktail». Left. We previously demonstrated that ω-3 PUFAs such as DHA and EPA as well as the ω-9 MUFA OA significantly inhibits HER2 expression and/or activity in human cancer models naturally exhibiting HER2 gene amplification and HER2 protein overexpression. Conversely, the ω-6 PUFA LA was found to significantly up-regulate HER2[34-39]. Right. Two prominent «fat features» of the «Mediterranean diet», i.e., a low ω-6/ω-3 PUFA ratio and elevated ω-9 MUFA levels appear to be a new composite biomarker of a low risk for breast cancer[40]. Since HER2 oncogene plays an active role in breast cancer etiology and metastatic progression, our findings support the notion that a low ω-6/ω-3 PUFA ratio and elevated ω-9 MUFA levels, the key «Mediterranean diet's fat features», can be expected to be extremely efficient at blocking HER2 expression. The predictable anti-HER2 actions of the «Mediterranean diet's fat features» not only can explain their potential protective effects on the promotion and progression of breast cancer disease.

features", can be expected to be extremely efficient at blocking HER2 expression (fig. 3).

In summary, considering that dietary interventions based on ALA-rich flaxseed has already been demonstrated to significantly reduce HER2 expression in breast cancer patients[8], our current characterization of the anti-HER2 actions of the ω-3 PUFA reveal an inexpensive and readily available dietary alternative or adjunct to currently used drugs in the management of HER2-positive breast carcinomas. This ALA-based therapeutic approach, which will repress HER2 at the transcriptional level, might be even more beneficial when given in combination with molecular therapies directed against HER2 such as trastuzumab, which target the ectodomain of HER2 and promote its degradation. Certainly, an appropriate dietary intervention reproducing the predictable anti-HER2 actions of the «Mediterranean diet's fat features» should be accomplished in animal models and human pilot studies in the future. Only then we will know whether the old «Mediterranean dietary traditions» can successfully work as a valuable «anti-HER2 cocktail» in the management of HER2 positive-breast cancer disease.

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